Cloning and characterization of the eae gene from a dog attaching and effacing *Escherichia coli* strain 4221

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Received 24 January 1997; accepted 27 January 1997

**Abstract**

We have cloned and determined the nucleotide sequence of the eae gene from a dog attaching and effacing (A/E) *Escherichia coli* (DEPEC) strain 4221. When comparing the predicted amino acid sequence of the eae<sub>DEPEC</sub> to that of the Eae proteins from enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* O157:H7 (EHEC), *Citrobacter freundii* biotype 4280, and a swine A/E *E. coli* strain O45 (PEPEC), the overall sequence identity was 84, 81, 83 and 83%, respectively, with the greatest divergence at the C-terminal end, the putative receptor-binding portion. Interestingly, the DEPEC Eae shares the greatest identity at the C-terminal region with the *Citrobacter freundii* Eae protein. We have constructed and purified a maltose-binding fusion protein (MBP) containing the product of the entire eae gene of the DEPEC strain 4221. Binding of MBP-Eae<sub>DEPEC</sub> fusion protein to HEp-2 cells was demonstrated by immunofluorescence microscopy. In addition, the Eae protein of DEPEC (4221) demonstrated a strong serological relationship with that of EPEC (E2348/69) as observed using a polyclonal antiserum against MBP-Eae<sub>DEPEC</sub> fusion protein.

**Keywords**: A/E *Escherichia coli*; Dog EPEC (DEPEC); eae; Fusion protein; HEp-2 cell

1. **Introduction**

The production of A/E lesions is a characteristic feature of A/E *Escherichia coli* (AEEC). In these lesions, intimate bacterial attachment to the intestinal brush border, followed by destruction of microvilli and distortion of the apical enterocyte membrane into an actin-rich pedestal structure at the point of bacterial contact are observed [1]. Human enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are AEEC. A/E lesions have also been associated with diarrhea in a variety of animal species, including rabbits, pigs, calves, lambs, horses and cats [2,3]. Signal transduction, resulting in induction of tyrosine phosphorylation of a 90-kDa host protein (Hsp90) and increased intracellular Ca<sup>2+</sup> levels has been associated with A/E lesion formation [4].

Several genes located in a 35-kb region of the chromosome (LEE region: locus of enterocyte effacement) are required for the expression of the A/E phenotype in EPEC strain E2348/69. The virulence genes contained in this region include the eae, espA, and espB genes, as well as the sep genes which are necessary for secretion of extracellular proteins (in-
cluding the EspA and EspB proteins) by EPEC [5,6]. The eae gene encodes intimin, a 94-kDa outer membrane protein, which is necessary for the intimate attachment of bacteria to host cell surface [7]. Mutation of the eae locus results in loss of the ability to produce the attaching and effacing effect [8] and the purified Eae protein shows the binding activity to HEP-2 cells [9]. The espA and espB genes, located downstream of eae on the chromosome, encode extracellular factors involved in signal transduction in epithelial cells [6,10]. The LEE region is also present in EHEC strains, the rabbit EPEC strain RDEC-1, strains of Citrobacter freundii, Hafnia alvei associated with diarrhea disease, and some A/E strains of swine E. coli [5,11].

Natural infections with AEEC in dogs have also been described [12]. Colony hybridization with DNA probes indicated that canine AEEC isolates do not carry enterotoxin, verotoxin or fimbrial genes common to enterotoxigenic E. coli and verotoxigenic E. coli, but they carry nucleotide sequences related to the EAF sequence and the bfpA, eae, espB genes [13]. Canine AEEC (DEPEC) strain 4221 was isolated from a dog with typical A/E lesions in the small intestine [13]. DEPEC strain 4221, which was positive for eae, espB, bfpA, and EAF, adhered to HEP-2 cells in a localized manner and was positive in the fluorescence actin staining test. In this study, we report the cloning and nucleotide sequence of the eae homologue from DEPEC strain 4221 and the characterization of its binding properties using a maltose-binding fusion protein (MBP)-EaeDEPEC.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain 4221 isolated from the intestinal contents of a dog with diarrhea and typical A/E lesions was used in this study [13]. The pig A/E E. coli strain 86-1390 (PEPEC, Pig EPEC), the human EPEC strain E2348/69 (O127:H6), and a rabbit diarrheagenic strain RDEC-1 (O15:NM) were used for the study of the antigenic relatedness of the Eae proteins [14]. The E. coli laboratory strain XL1-Blue (Stratagene, La Jolla, CA) and HB101 [15] were used as host for recombinant plasmids. Plasmid pCR®.II vector (Invitrogen Corporation, San Diego, CA) was used for cloning the PCR products. The pMAL-p2 vector (New England Biolabs Inc., Beverly, MA) was used for cloning the entire fragment containing the eaeDEPEC gene.

2.2. Cloning and sequencing of the eaeDEPEC

Based on our previous observation that DEPEC strain 4221 contained sequence homologous to EPEC eae gene [13], FMI (5′CATATGGAAAGGAGAGT3′) annealed at 2268–2287 nt and RYu4 (5′ATCTTCTGCTACTGCTTC3′) annealed at 3038–3058 nt of the EPEC eae gene, were used to generate an internal 0.8 kb fragment of eaeDEPEC sequence. The amplified fragment was cloned into the pCR®.II vector and sequenced. The rest of the eaeDEPEC gene was cloned using the methods described by Yu and Kaper with some modifications [16]. Briefly, chromosomal DNA from strain 4221 was digested with XhoII and ligated under dilute conditions (0.01 µg/ml) in order to have self-ligation. Circular DNA molecules were then used as a template for inverse PCR. Two primers, MAB1 (5′CATAGAACGGTAATAAG3′) annealed at 2333–2349 nt and MAB2 (5′CGAGCCAGATATGCTAAACAG3′) annealed at 2900–2921 nt of the EPEC eae gene, were used to generate a 4 kb fragment covering the N-terminus and C-terminus of the eaeDEPEC gene. The amplified fragment was then cloned into pCR®.II vector and sequenced. The nucleotide sequence was determined by the dyeox chain termination technique using a Sequencing® Kit (Pharmacia LKB Biotechnology Inc., Bai d’Urfé, Quebec, Canada) according to the procedure recommended by the manufacturer. DNA sequence analyses were performed using the GeneWorks program (IntelliGenetics, Inc., CA) and programs included in the GCG (Genetics Computer Group, Madison, WI) package. The deduced amino acid sequence was used to search the combined databases of the National Center for Biotechnology Information (Washington, DC) via the BLAST network service. The database searches employed the alignment algorithm of Altschul et al. [17]. The nucleotide sequence of the DEPEC strain 4221 eae gene has been submitted to GenBank database and assigned the accession number U66102.
2.3. PCR and enzymatic amplification

For PCR, 50 ng of DNA was mixed with 2 U of Taq polymerase, 200 mM deoxynucleotide triphosphate, 0.5 μM primers, and 1× buffer (10× buffer is 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 mM MgCl₂ to a final concentration of 3 mM) in a final volume of 50 μl [14]. The reaction mixtures were overlaid with oil. The samples were heated at 80°C for 5 min (hot start) after the Taq polymerase (Gibco BRL, Burlington, Ont., Canada) was added, and the samples were subjected to PCR in a thermal cycler. The program used was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles, followed by a final 7 min extension at 72°C.

2.4. Northern (RNA) blotting

Total RNA from Luria broth early-exponential phase culture of DEPEC strain 4221 was isolated using TRIZol® reagent according to the manufacturer’s recommendations (Gibco BRL). The hybridization was carried out by the standard procedures [18] using nylon membrane filters. Prehybridization was performed for 6 h at 42°C and hybridization was carried out overnight at 42°C with the PCR-generated 3 kb eae fragment labelled with [α-32P]dCTP by a random primer method (Pharmacia LKB). The filter was washed with 1× SSC-1% SDS at room temperature twice and three times in 0.1× SSC-0.1% SDS at 65°C, then exposed to X-ray film (Kodak, Rochester, NY) at −70°C with an intensifier screen.

2.5. Construction and purification of MBP-EaeDEPEC fusion protein

Two eaeDEPEC specific primers, AFB (5'-GGATCCACTACTCATCTCAACTC-3') and ARX (5'-TCTAGAAGTAGACATAAAG-3’), were designed to amplify the open reading frame (ORF) encoding the EaeDEPEC protein by PCR. Primers AFB and ARX included a BamHI and a XbaI restriction site, respectively. The amplified fragment covering the entire eae DEPEC gene was cloned into pMAL-p2 plasmid and transformed into E. coli HB101.

The EaeDEPEC was expressed as MBP-EaeDEPEC fusion protein using the pMAL-p2 expression system (New England Biolabs). The bacteria were induced in the presence of 1 mM isopropyl-β-d-thiogalactoside (IPTG) at an OD₆₀₀ of about 0.5. 3 h after induction, cells were harvested, and the fused protein was purified using an amylose resin column (New England Biolabs). The eluted fusion protein was characterized by SDS-PAGE and immunoblotting with anti-MBP antiserum (New England Biolabs) [19] and used for immunization.

2.6. Production of polyclonal anti-MBP-EaeDEPEC antibody and detection of EaeDEPEC, EaeDEPEC, EaeRDEC-1, and EaeDEPEC proteins

Polyclonal antibody against purified MBP-EaeDEPEC fusion protein was produced using a New Zealand White rabbit as described previously [19]. The serological relationship of the Eae proteins in whole-cell extracts of DEPEC strain (4221), PEPEC strain (86-1390), RDEC-1 and EPEC strain (E2348/69) was examined in Western blot using the polyclonal antiserum produced against MBP-EaeDEPEC protein (dilution 1:2000) as described previously [19].

2.7. Binding of the MBP-EaeDEPEC fusion protein to HEp-2 cells

Binding assays of the MBP-EaeDEPEC fusion protein to HEp-2 cells were performed as previously described with modifications [9]. Briefly, HEp-2 cells were grown to confluence in eight-chambered slides (Nunc Inc., Naperville, IL) containing Dulbecco’s modified Eagle’s medium (Gibco BRL) with 10% fetal bovine serum (Gibco BRL), 100 U of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a humidified atmosphere containing 5% CO₂. Dulbecco’s modified Eagle’s medium was replaced with 200 μl of RPMI-0.4% bovine serum albumin (BSA)-20 mM HEPES (pH 7.0)-2.5 mM maltose (binding solution), and cells were incubated for 45 min at 37°C in the presence of 5 μg of MBP-EaeDEPEC fusion protein or of MBP as a negative control. The cells were washed five times with phosphate-buffered saline (PBS). Rabbit anti-MBP antiserum was diluted 1:1000 in binding solution, and 250 μl was added to each chamber. The cells were incubated for 30 min at 37°C and then washed five times
with PBS. Fluorescence-labelled porcine antirabbit antibody was diluted 1:1000 with binding solution, and 250 µl was added to each chamber. After 30 min of incubation at 37°C, the cells were washed four times with PBS and fixed in 95% ethanol-5% acetic acid at −20°C for 15 min. Slides were then examined by UV microscopy.

3. Results and discussion

3.1. Nucleotide sequence analysis of the DEPEC strain 4221 eae gene

An amplified 0.8 kb fragment generated by PCR using primers FM1 and RYu4 corresponding to an internal region of eae was cloned into the pCR® II vector and sequenced. Primers (MAB1 and MAB2) derived from the internal sequence of eaeDEPEC were used to obtain a 4 kb inverse PCR fragment on XhoII digested chromosomal DNA of DEPEC strain 4221. This 4 kb fragment was then cloned into the pCR® II vector and sequenced. The sequence obtained from these clones represented the entire DEPEC strain 4221 eae gene. Nucleotide sequence analysis of the eae gene from DEPEC strain 4221 revealed an open reading frame (ORF) of 2820 bp which could potentially encode a protein of 940 residues with a predicted molecular mass of 102.6 kDa. A 2.9 kb eaeDEPEC specific transcript detected from Luria broth culture of the DEPEC strain 4221 in the early exponential phase by a Northern blotting con-

3.2. Sequence homology among eae genes from DEPEC, EPEC, EHEC strains and Citrobacter freundii biotype 4280

The greatest homology among the eaeDEPEC, eaeEPEC, eaeEHEC, eaeCI, and eaePEPEC genes was found in the N-terminus of the sequences whereas a significant sequence divergence was found in the C-terminus of the sequences. Table 1 shows the alignment of the predicted Eae proteins of DEPEC, EPEC, EHEC, C. freundii and PEPEC. The carboxy-terminal 240 residues of the predicted polypeptide of the DEPEC strain 4221 exhibited a low degree of homology to EPEC Eae, EHEC Eae, and PEPEC Eae and the highest degree of homology to C. freundii Eae.

3.3. Construction and purification of MBP-EaeDEPEC fusion protein and binding of the expressed MBP-EaeDEPEC to HEp-2 cells

The structural gene of mature EaeDEPEC peptide was expected to be expressed as a MBP-EaeDEPEC fusion protein with a molecular mass of 142 kDa. SDS-PAGE analysis of the whole-cell lysates indicated that a protein of approx. 142 kDa was expressed (data not shown). After purification by affinity chromatography on an amylose column, 5 mg of